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Tailoring Gut Microbiota for Enhanced Resilience and Performance under Sleep-Deprived Conditions.

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14. Abstract:
Sleep deprivation has been associated with an increase in overall mortality and morbidity. Mechanistically, a loss of brain mass, due to neuronal death, has been observed after chronic sleep deprivation. Sleep deprivation also has enormous impacts on metabolic homeostasis, with increased prevalence of weight gain and obesity. Interestingly, perturbation of gut microbiota presents a pattern of metabolic abnormalities mirroring those induced by sleep deprivation. In this study, perturbation of gut microbiota and host functions following sleep deprivation will first be determined, using a humanized rat model, which is derived by transplanting native human gut microbiota into germ-free rats. This microbiota perturbation effect is based upon our hypothesis that sleep deprivation initially causes degradation in the functional and structural integrity of the gastrointestinal tract. Data generated will be used to develop a predictive metabolic network model of host-microbiota interaction. This model will be validated under various conditions of gut microbiota modulations using prebiotic, probiotic and synbiotic approaches. The validated model will be used to identify targets that can effectively tailor the gut microbiota and counter the adverse effects of sleep deprivation, resulting in enhanced host resilience and performance. This study is a four year project, the current report describes the accomplishments during the first year including establishment of the rodent germ-free facilities, selection of the human donors, and the creation of the pseudo germ-free animal model. This report also describes the work plan and future direction of the project.

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PREFACE

The studies were conducted at the Molecular Bioeffects branch (RHDJ), Bioeffects Division (RHD), Airman Systems Directorate of the 711 Human Performance Wing of the Air Force Research Laboratory (AFRL/711 HPW/RHDJ), Wright-Patterson AFB, OH. This interim technical report covers the performance period June 1, 2014 – Sep 30, 2015 and was written for AFRL Work Unit H0AP.

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All studies involving animals were approved by the Wright-Patterson Institutional Animal Care and Use Committee, and were conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International, in accordance with the Guide for the Care and Use of Laboratory Animals, National Research Council (2011). Studies were conducted under approved Air Force Research Laboratory Institutional Animal Care and Use Committee, animal protocol number F-WA-2015-0156-A.

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Summary

Sleep deprivation has been associated with an increase in overall mortality and morbidity. Mechanistically, a loss of brain mass, due to neuronal death, has been observed after chronic sleep deprivation. Sleep deprivation also has enormous impacts on metabolic homeostasis, with increased prevalence of weight gain and obesity. Interestingly, perturbation of gut microbiota presents a pattern of metabolic abnormalities mirroring those induced by sleep deprivation. The importance of the gut microbiome in metabolic disorders such as obesity and diabetes has been demonstrated and certain mixes of gut microbiota may protect or predispose the host to obesity. For example, a shift towards microbial genus *Firmicutes* from *Bacteroidetes* has been observed in obese individuals. In contrast, if an obese individual loses weight, a shift towards *Bacteroidetes* occurs. Despite the similar patterns of metabolic disorders induced by perturbation of gut microbiota and sleep deprivation, it is not clear if there is any interaction between these two conditions.

In this study, perturbation of gut microbiota and host functions following sleep deprivation will first be determined, using a humanized rat model, which is derived by transplanting native human gut microbiota into germ-free rats. This microbiota perturbation effect is based upon our hypothesis that sleep deprivation initially causes degradation in the functional and structural integrity of the gastrointestinal tract. Data generated will be used to develop a predictive metabolic network model of host-microbiota interaction. This model will be validated under various conditions of gut microbiota modulations using prebiotic, probiotic and synbiotic approaches. The validated model will be used to identify targets that can effectively tailor the gut microbiota and counter the adverse effects of sleep deprivation, resulting in enhanced host resilience and performance. Therefore, knowledge gained from this study will significantly facilitate future development of novel strategies for enhanced resilience and performance optimization in warfighters under sleep deprived or other stressful conditions.

This study is a four year project, the current report describes the accomplishments during the first year including establishment of the rodent germ-free facilities, selection of the human donors, and the creation of the pseudo germ-free animal model. This report also describes the work plan and future direction of the project.

1.0 Introduction

Sleep deprivation, sleep restriction, and circadian desynchrony are among the most commonly encountered stressors in sustained military operations. Overall insufficient sleep leads to fatigue, sleepiness, impaired mood regulation, and cognitive and physical performance degradation (Turner et al, 2007; Mograss et al, 2009; Alhaider et al, 2011; Aleisa et al, 2011; Alhaider et al, 2010; McDermott et al, 2003; Guan et al, 2004). Animal model studies found a loss of brain mass occurring after chronic sleep deprivation due to neuronal cell death, even in the presence of environmental enrichments (Mirmiran et al,

1986; Morrissey et al, 2004). This may be due to the accumulation of misfolded proteins and subsequently activation of the apoptotic pathway (Cirelli et al. 2004; Mackiewicz et al., 2007; Cirelli and Tononi, 2008; Cirelli and Bushey, 2008; Thompson et al, 2010).

Sleep deprivation has enormous impacts on metabolic homeostasis, leading to increased risk of a range of metabolic disorders, including cardiovascular and gastrointestinal morbidity, peptic ulcers, obesity, insulin resistance, and type 2 diabetes. A relationship between sleep deprivation and prevalence of weight gain and obesity has been shown (Bollinger et al, 2009). Interestingly, perturbation of gut microbiota presents a pattern of metabolic abnormalities mirroring those associated with sleep deprivation. The importance of the gut microbiome in metabolic disorders such as obesity and diabetes has been demonstrated and certain mixes of gut microbiota may protect or predispose the host to obesity. A shift in gut microbiotia populations towards genus *Firmicutes* from *Bacteroidetes* has been observed in obese individuals. In contrast, if an obese individual loses weight, a shift towards *Bacteroidetes* occurs (Cryan & Dinan, 2012). Despite the similar patterns of metabolic disorders induced by perturbation of gut microbiota and sleep deprivation, it is not clear if there is any interaction between these two conditions.

Studies in obese populations provide important clues to the connection between sleep deprivation and perturbation of gut microbiota. Obesity, in part, is characterized by systemic inflammation, in which the brain-gut axis is likely to be involved (Tahergorabi and Khazaei, 2013). While it is not clear how this inflammatory response is activated, sleep deprivation could compromise the functional and structural integrity of the gastrointestinal tract. On one hand, changes in intestinal functions cause a perturbation in the gut microbiota composition and metabolism. On the other hand, degradation in the structural integrity results in increased intestinal permeability, and subsequently, the leakage of gut microbial cells both externally and interstitially, causing exposure to degrading factors (i.e. lipopolysaccharides (LPS)). This degraded epithelial state in turn triggers mucosal and systemic inflammation. Jazwinska reported that the proliferation of jejunum cells, which function to restore cell loss in this region, was down-regulated after sleep deprivation (Jazwinska, 1986). If this is proven to be a wide-spread phenomenon throughout the intestine following sleep deprivation, it would provide a mechanistic basis for the observation that bacteria migrated from the intestine and translocated to extra-intestinal sites, may result in systemic bacterial invasion and infection following sleep deprivation (Everson and Toth, 2000).

While sleep deprivation results in a dysregulated hypothalamus-pituitary-adrenal gland (HPA) axis and increased cortisol levels (Aldabal & Bahammam, 2011), stress is known to increase intestinal permeability, allowing gut microbiota an opportunity to translocate across the intestinal mucosa and directly interact with immune cells (Gareau et al, 2008; Teitelbaum et al, 2008). An exaggerated HPA response to restraint stress in germ-free (GF) mice, compared with specific pathogen-free (SPF) mice, further confirmed that gut microbiota plays an important role in HPA regulation (Sudo et al, 2004). Consistently, probiotics has been shown to prevent increases in intestinal permeability and HPA axis hyper-reactivity, induced by maternal separation and restraint stress (Gareau et al, 2007;

Ait-Belgnaoui et al, 2012). A recent study showed that sleep deprivation and high calorie, high fat Western diet act additively on the CNS (Alzoubi et al, 2013).

After combining the findings of the research in sleep/sleep deprivation, gut microbiota, metabolic diseases and psychological disorders, we have developed a hypothesis that sleep deprivation initially degrades the functional and structural integrity of the gastrointestinal tract (and other organs including the brain), resulting in altered metabolic homeostasis and intestinal permeability. While the former induces perturbation of the gut microbiota, the latter results in the leakage of microbial cells and factors (i.e. LPS). The combined effects of these events can trigger mucosal and systemic inflammation, activation of HPA axis and increased levels of cortisol. A simplified version of this hypothesis (for the ease of visualization) is depicted in the figure below.

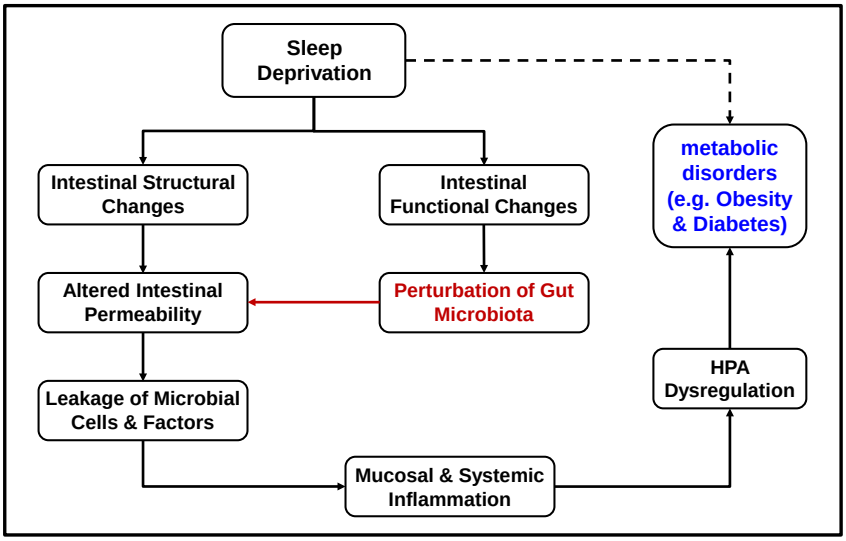

Figure1. Representation of effects of sleep deprivation in HPA axis dysregulation

To test this hypothesis and to study the effects of gut microbiota modulation, we propose to:

1) Investigate the effects of sleep deprivation on gut microbiota and host functions using a gnotobiotic rat model harboring the human gut microbiota (i.e. a “humanized” rat model); and
2) Investigate the feasibility of enhancing host resilience to counter the adverse effects of sleep deprivation by modulation of the gut microbiota.

To achieve these objectives, we will first develop a new humanized rat model harboring the gut microbiota from five-ten healthy normal human donors. This model will have a better representation and coverage of the entire spectrum of the human gut microbiota. Using this new animal model, we will investigate the effects of sleep deprivation on gut microbiota population and metabolism, as well as the effects on host intestinal and systemic functions. These results will be used to develop a host-microbiota metabolic

interaction network model. The model developed will be used to identify targets and strategies for modulating gut microbiota to counter the adverse effects of sleep deprivation and enhanced resilience and performance in the host. The effect of gut microbiota modulation will first be tested *in silico*. The most effective strategy will be selected for experimental validation.

2.0 Methods

2.1 Experimental Approach

Initially, a pseudo germ-free animal model will be created by using a four antibiotic cocktail to swipe the native rat microbiome. Then, fecal microbiota collected from normal healthy human donors will be used to inoculate pseudo germ-free (GF) Sprague Dawley (SD) rats to develop the humanized rat model. Due to the fact that GF rats are no longer commercially available in the US, pseudo GF rats, generated by antibiotic treatment of specific-pathogen-free rats, will be used. Successful establishment of the transplanted human microbiota in the animals will be confirmed by community fingerprinting of the PCR products of the microbial V1-V3 region of the16S rRNA gene. The rats derived from individual donors will then be co-housed to establish a unified de-individualized humanized rat (DHR) model.

To investigate the effect of acute and chronic sleep deprivation on gut microbiota, animals of the DHR model will be subjected to either acute or chronic sleep deprivation. Cecal contents from sleep deprived animals will be collected and profiled using metagenomics and metatranscriptomics techniques to determine the composition of the microbiota community, the metabolically active members and the collective metabolic profiles of the microbiota community.

An integrated approach to examine the metabolic activities of the gut microbiota will be performed to study the biological significance of microbiota perturbation following sleep deprivation. To further confirm the effects of differential gene expression, changes in protein levels, as well as changes in metabolite levels resulting from protein expression changes, will be confirmed by targeted proteomics and metabolomics, using LC/MS-MS.

Sections of the small and large intestine and mesenteric lymph nodes will be isolated, and changes in the host intestinal functions investigated. Blood samples will be collected from these animals and used in the analyses of serum profiles of selected hormones, endotoxin load and cytokines for the assessment of HPA axis dysregulation and systemic inflammation.

The biological significance of microbiota metabolic perturbation will be inferred using the biological association network analysis. The biological association networks of dysfunctions in host (i.e. compromised intestinal functions and permeability, HPA axis dysfunction, and mucosal/ systemic inflammation) will be generated using the Ingenuity

Pathway Analysis tool. These networks will be further enriched by combining the results of the STRING and STITCH database searches.

Safe and effective strategies will be developed to functionally reverse or compensate the perturbation of the microbiota metabolism and the changes in the host-microbiota interaction. The feasibility of tailoring gut microbiota to generate a new homeostatic state(s) for enhanced resilience in the host will be explored. The synbiotic effects of combinations of prebiotics and probiotics will first be evaluated *in silico* using the host-microbiota metabolic interaction network model, followed by experimental testing to ensure that the desired outcome will be achieved.

3.0 Results

3.1 Collection and characterization of human fecal samples

During this period of time, we have obtained the fecal materials from six human donors through OpenBiome (Somerville, MA). These samples have been characterized using the metagenomic technique of 16S ribosomal RNA sequencing. The sequencing data suggested that samples from five donors were found to be suitable for transplantation into germ-free rats. We also established the germ-free facility and the sleep deprivation model that is compatible with this germ-free environment. Molecular, biochemical and cellular assays are being developed for determining the changes in microbiota community and functions, and host intestinal, immune and HPA functions based on the AFOSR-funded sleep deprivation study. We are currently developing a procedure for generating pseudo germ-free rats in a reproducible manner using a four-antibiotic cocktail. Once the effectiveness of this procedure is confirmed, it will be used to generate pseudo germ-free rats for the development of the gut microflora humanized rat model.

The contract with OpenBiome for the collection of fecal samples from normal human subjects was established in March, 2015. Subjects were recruited under very restrictive inclusion/exclusion criteria. Some of these requirements are outline below.

- Male
- Caucasian
- 20-45 years old
- Normal body weight (BMI 19-25)
- No drug abuse
- Good health condition
    - No antibiotic use for at least 12 months
    - Not under any medication for at least 12 months
    - No infectious, gastrointestinal, immunosuppressive, autoimmune, psychological, neurological and sleep disorders.

Six human fecal samples were subsequently received from OpenBiome. Initially, the titer of these samples was determined, and the results are shown in the Table 1 below. The

titer of these samples ranged from 1.1 – 8.1 x10^8 cfu/g. As expected, the titer of anaerobes is significantly higher than that of aerobes in all of these samples ($p < 0.01$).

Sample ID	Titer (cfu/g)		
	Aerobic	Anaerobic	Total
26_049	3.7E+07	7.70E+08	8.1E+08
28_002	9.3E+05	4.18E+08	4.2E+08
37_044	2.9E+06	1.11E+08	1.1E+08
60_014	7.8E+05	1.97E+08	2.0E+08
65_011	2.6E+06	1.39E+08	1.4E+08
72_003	3.3E+06	3.54E+08	3.6E+08

Table 1. Representation of the bacterial titers from selected six donors

These samples were characterized by 16S rRNA sequencing (i.e. metagenomics). It was found that these donors have different ratios of *Bacteroidetes* to *Firmicutes*. Although the average ratio of *Bacteroidetes* to *Firmicutes* of this small subject population is approximately 1:1, two donors showed *Bacteroidetes* dominant, two have similar levels of *Bacteroidetes* and *Firmicutes*, and the remaining two showed *Firmicutes* dominant (see figures below).

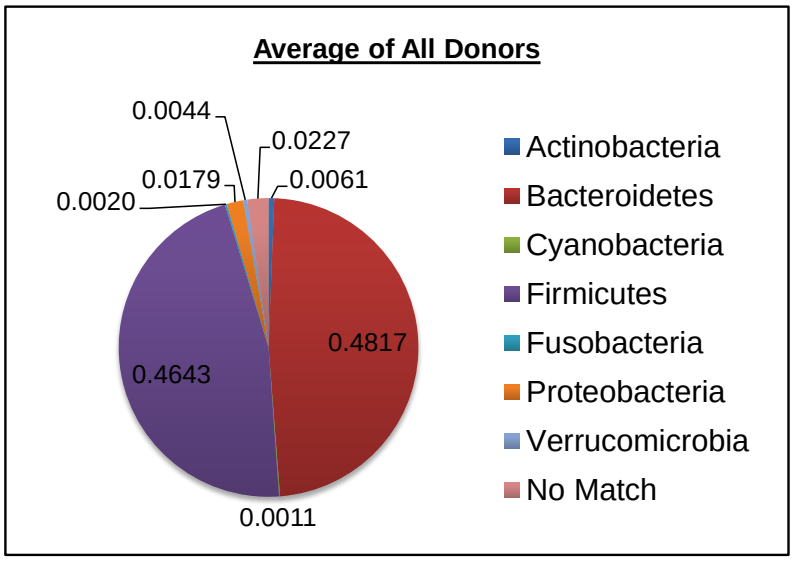

Figure 2. Identification of different ratios of bacteria from human donor samples characterized using 16S rRNA sequencing

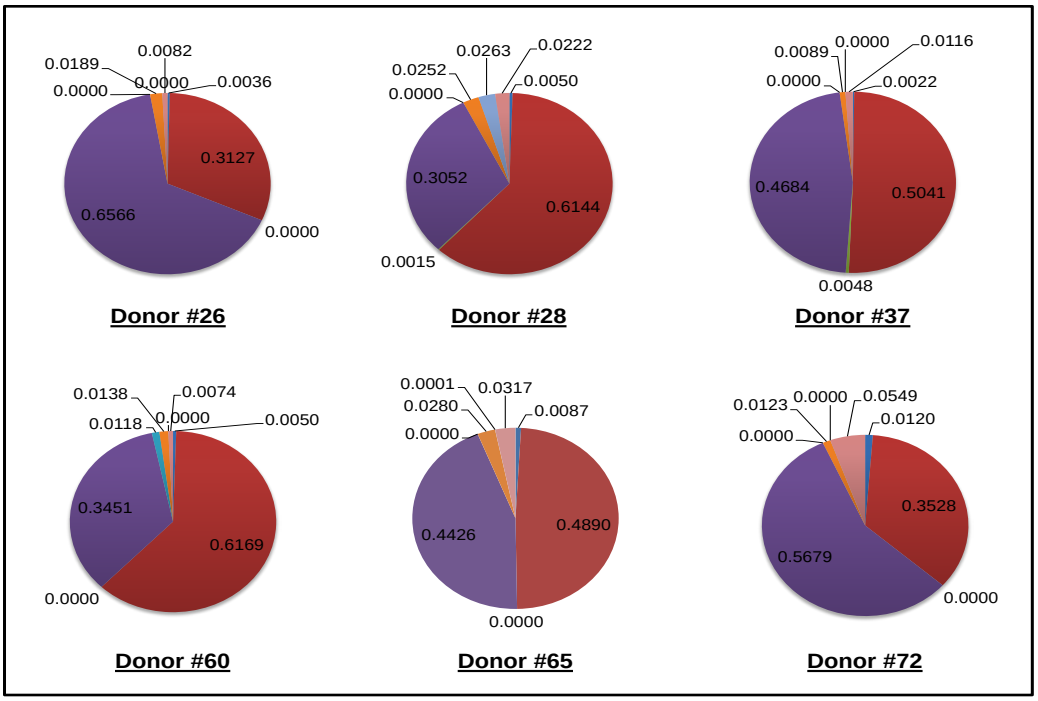

Figure 3. Identification of bacterial ratios of each individual donor

Bacteroides and *Parabacteroides* of *Bacteroidetes*, and *Faecalibacterium*, *Megamonas*, *Dialister* and *Blautia* of *Firmicutes* are among the most common genera in the fecal microbiota in these human donors. Additional analysis of the sequencing data revealed that Donor #28 has a slightly different profile of the top 10 genera, compared to other donors, especially Donor #60 (who also shows *Bacteroidetes* dominant). Donor #28 showed high levels of bilirubin in all five laboratory test screens, and was subsequently diagnosed as Gilbert's Syndrome. Because of this diagnosis, this donor will be excluded from the fecal transplant experiment for the development of the humanized rat model. Operational taxonomic unit (OTU) are used to characterize bacteria based in sequence similarity of the 16SrRNA. The top 10 genera (family) identified by OTU after 16SrRNA sequencing in the fecal microbiota among the human donors are shown in Table 2 below.

OTU ID	Donor ID						Average %	Taxonomies (Phylum; Class; Order; Family; Genus; Species)
	26_049	28_002	37_044	60_014	65_011	72_003		
4463892	8.48%	14.72%	23.79%	9.27%	20.32%	19.90%	16.08%	Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides; -
4478125	9.63%	1.48%	8.26%	1.97%	2.78%	5.69%	4.97%	Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Faecalibacterium; prausnitzii
4445028	7.44%	0.40%	2.57%	2.05%	1.72%	6.57%	3.46%	Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Faecalibacterium; prausnitzii
4467447	1.91%	3.87%	3.45%	10.68%	1.13%	5.91%	4.49%	Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides; -
4457438	7.87%	0.00%	0.60%	0.25%	9.32%	2.25%	3.38%	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; -; -
4447950	0.00%	1.72%	0.00%	25.55%	16.51%	0.00%	7.30%	Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides; -
4483963	13.71%	0.00%	0.00%	4.20%	0.00%	0.00%	2.99%	Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides; -
4401375	0.00%	12.02%	0.25%	0.68%	3.57%	0.00%	2.75%	Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides; uniformis
4447072	2.15%	0.21%	10.19%	0.06%	0.20%	0.00%	2.13%	Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides; -
4457872	1.05%	0.31%	3.54%	0.33%	0.00%	2.34%	1.26%	Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides; -
1952	0.24%	2.97%	1.19%	0.30%	2.16%	0.47%	1.22%	Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Parabacteroides; -
4449054	0.00%	0.78%	2.97%	1.00%	1.81%	0.00%	1.09%	Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides; -
2530636	0.00%	0.00%	0.00%	11.11%	0.00%	30.49%	6.94%	Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Megamonas; -
4480244	2.48%	0.00%	0.00%	0.49%	8.08%	0.66%	1.95%	Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Dialister; -
4465907	1.26%	2.75%	0.29%	0.47%	0.28%	1.39%	1.07%	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Blautia; -
171772	0.00%	5.41%	0.00%	0.00%	0.00%	0.00%	0.90%	Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae;
4377149	0.00%	0.00%	5.16%	0.00%	0.00%	0.00%	0.86%	Firmicutes; Clostridia; Clostridiales;
4451152	0.00%	0.00%	0.00%	5.08%	0.00%	0.00%	0.85%	Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides;
3138798	0.00%	4.23%	1.24%	1.31%	0.00%	0.00%	1.13%	Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Phascolarctobacterium;
206233	0.00%	4.19%	0.00%	0.00%	0.00%	0.00%	0.70%	Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides;
4453609	1.33%	3.20%	0.00%	0.76%	0.00%	0.00%	0.88%	Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae;
4474593	0.02%	0.24%	0.00%	0.00%	0.32%	3.27%	0.64%	Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides; fragilis
4443172	2.97%	0.00%	0.07%	0.59%	0.40%	0.00%	0.67%	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae;
4306262	0.00%	2.63%	0.00%	0.00%	0.00%	0.00%	0.44%	Verrucomicrobia; Verrucomicrobiae; Verrucomicrobiales; Verrucomicrobiaceae; Akkermansia; muciniphila
4346675	1.16%	0.00%	2.61%	0.49%	0.76%	0.00%	0.84%	Firmicutes; Clostridia; Clostridiales; Ruminococcaceae;
3039313	2.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.34%	Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Megasphaera;
2201995	0.00%	0.38%	0.00%	0.00%	1.81%	0.00%	0.36%	Proteobacteria; Betaproteobacteria; Burkholderiales; Alcaligenaceae; Sutterella;
4479443	0.99%	0.46%	1.04%	1.74%	0.59%	0.65%	0.91%	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae;
4484075	0.80%	0.00%	0.00%	1.52%	0.00%	0.00%	0.39%	Firmicutes; Clostridia; Clostridiales; Ruminococcaceae;
4413347	0.31%	0.37%	0.20%	0.40%	0.74%	1.12%	0.52%	Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; Bifidobacterium;
No Match	0.82%	2.22%	1.16%	0.74%	3.17%	5.49%	2.27%	-; -; -; -; -; -

Table 2. Representation of diverse bacterial families from human fecal donors identified (OTU). The yellow boxes represent the percentage of the top ten OTUs for each individual donor. The average % column represent the percentage of bacteria in all six donors identified by OTU.

A technique for community fingerprinting is also being established. Genomic DNA was extracted from these samples, and subjected to polymerase chain reaction (PCR) to amplify the 16S rRNA V1-V3 region using primers with a GC-clamp (Figure 4).

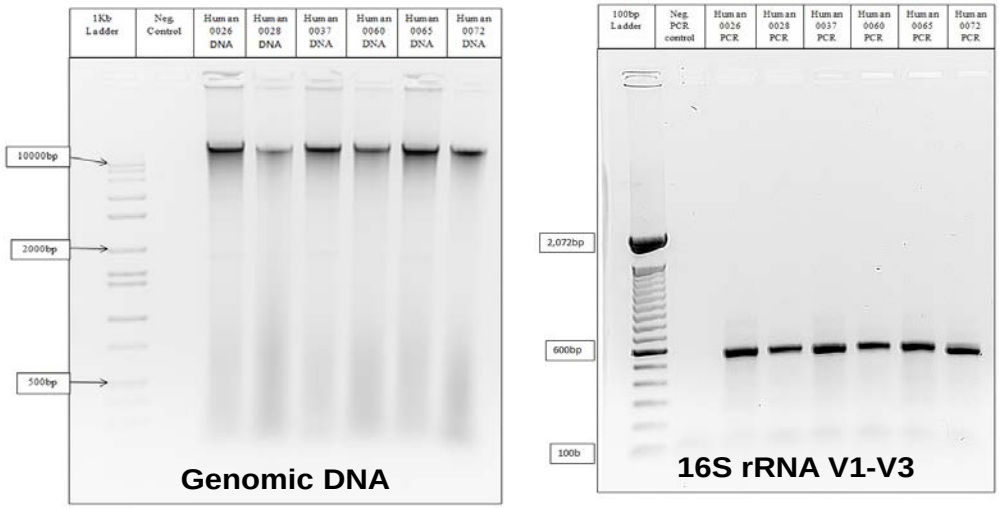

Figure 4. Gel electrophoresis of the genomic DNA and PCR amplification of bacteria from OpenBiome human donors

Community fingerprinting analysis of these PCR products will be performed using the temporal temperature gradient gel electrophoresis technique (TTGE).

3.2 Establishment of Germ-Free Facility

The animal facility for maintaining germ-free rats, and performing sleep deprivation experiments, was established in the vivarium at WPAFB. There are five semi-rigid germ-free isolators that provide a maximum capacity of 180 rats. This laboratory is fully functional and currently being used for generating/housing pseudo germ-free rats. The pictures of the germ-free laboratory and a germ-free isolator are shown in Figure 5 below.

Figure 5. Illustration of the rodent germ-free facilities to conduct microbiome studies at WPAFB, OH

3.3 Generating Pseudo Germ-Free Rats

We have developed a four-antibiotic cocktail for generating germ-free rats. The effectiveness of this cocktail was tested by microbial culture on agar plates using cecal content isolated from our previous sleep deprivation study (AFOSR funded). To minimize the potential side effects, relatively low levels of antibiotics were used. Additionally, these antibiotics were selected based on the fact that they cause minimal systemic exposure. As shown in the Table 3 below, combining these four antibiotics resulted in an effective regimen that can inhibit the growth of approximately 90% of the microbial community *in vitro*. Addition of a fifth antibiotic, ampicillin, to this cocktail only increased its effectiveness slightly.

Treatment Group	Colonies / Plate (Average)			% of Control		
	Aerobic	Anaerobic	Combined	Aerobic	Anaerobic	Combined
Control	**262**	**529**	**790**	**100%**	**100%**	**100%**
Enrofloxacin (500 µg/ml)	106	398	504	40%	75%	64%
Neomycin (150 µg/ml)	37	277	314	14%	52%	40%
Vancomycin (350 µg/ml)	86	632	718	33%	120%	91%
Amphotericin B (1000 µg/ml)	263	411	674	101%	78%	85%
4-Drug Cocktail	**8**	**89**	**97**	**3%**	**17%**	**12%**
Ampicillin (150 µg/ml)	230	316	546	88%	60%	69%
5-Drug Cocktail	**5**	**73**	**78**	**2%**	**14%**	**10%**

Table 3. Representation of bacteria colonies grown after treatment with individual drug, four and five cocktail drug combination.

The testing of the effectiveness of this four-drug cocktail in practice animals *in vivo* is in progress. Antibiotic treatment for ten days reduces the titers of cecal content by more than 95%. The results are shown in the Table 4 below.

Titer of Cecal Content (% of Day 0)		
Timepoint (Day)	**Control**	**Antibiotic-Treated**
0	100%	N/A
3	18%	18%
6	22%	12%
10	11%	3%

Table 4. Percentage of bacteria titer in cecal content after 10 days of antibiotic treatment with four drug cocktail (Enrofloxacin, Neomycin, Vancomycin and Amphotericin B)

4.0 Other technologies, assay developments and future plans

4.1 Sleep deprivation model compatible with germ-free isolators

In this study, sleep deprivation in animals will be achieved using the sleep fragmentation chambers maintained in germ-free isolators. Custom-modifications of the germ-free isolators have been made, and each isolator can accommodate up to 18 sleep fragmentation chambers. Thus, this experiment setup can effectively prevent cross-contamination of the gut microbiota between different experimental groups.

4.2 Molecular, biochemical and cellular assays

Assays for detecting changes in microbiota community and functions, and host intestinal immune and HPA functions following sleep deprivation, are being established using the samples collected from AFOSR-funded sleep deprivation study. Standard operation procedures (SPOs) developed in our laboratory (e.g. immunohistochemistry, enzyme-linked immunosorbent assay, and qRT-PCR, etc.) can be directly transitioned to this study. Assays that will be used in this study includes:

- Microbial community fingerprinting
- Metagenomics
- Metatranscriptomics (rRNA & mRNA)
- Colonic permeability
- Mucosal bacterial adhesion and penetration
- Mesenteric lymph nodes infection
- Serum bacteria-derived endotoxin load
- Mucosal inflammation
- Cytokine profile
- Intestinal regeneration
- Myenteric plexus neuronal excitability

- HPA axis function

4.3 Work Plan and Future Directions

The work plan for the next reporting period will be the development and characterization of the de-individualized humanized rat (DHR) model (Figure 6).

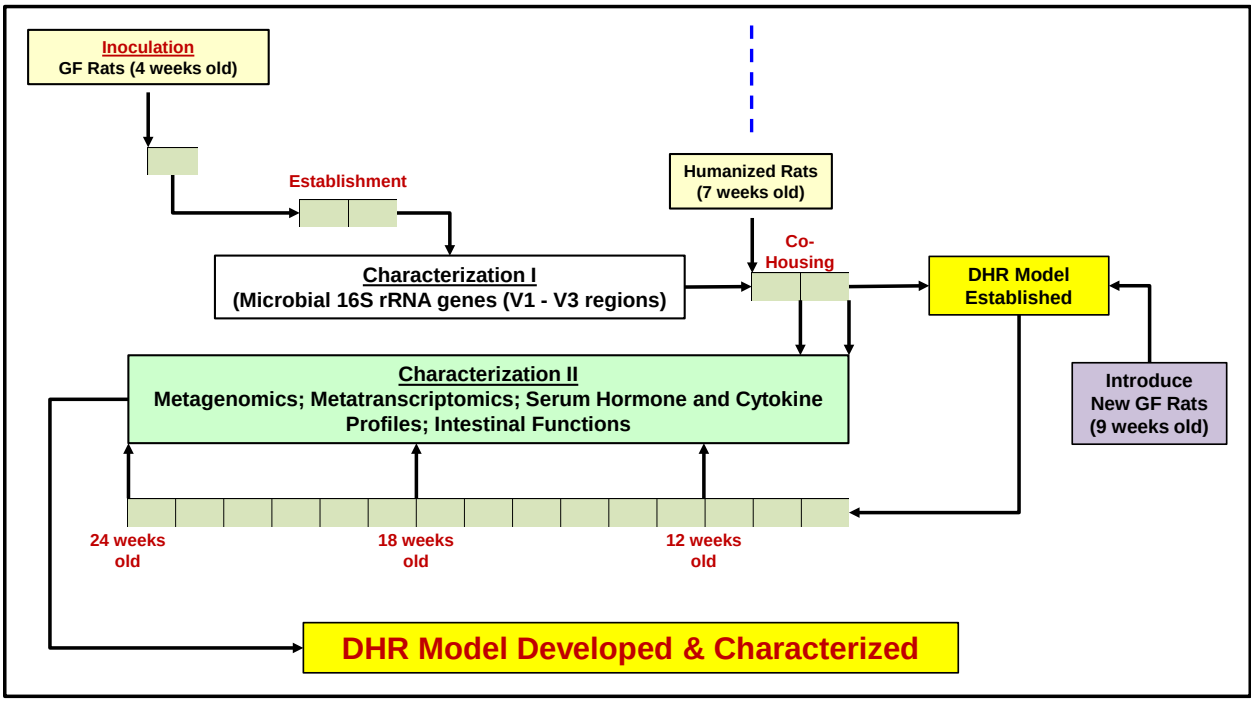

Figure 6. Graphical representation of the work plan to develop the de-individualized humanized rat (DHR) model to conduct microbiome studies.

This new gnotobiotic rat model harboring the "de-individualized" human gut microbiota will be developed using a two-step approach. The first step is to colonize GF animals using the microbiota derived from individual human donors. Subsequently, rats colonized with individual-specific microbiota will be allowed to socialize and transfer gut microbiota among themselves.

Fecal materials from human donors suitable for microbiota transplantation have been collected and identified. These samples will be used to inoculate GF SD rats twice, two days apart, by intragastric gavage. The animals will be maintained in a germ-free environment for two weeks to allow the human microbiota to establish and stabilize in the hosts. Successful establishment of the transplanted human microbiota in the animals will be confirmed by TTGE of the PCR products of the bacterial 16S rRNA genes. Samples from the animals receiving the microbiota from the same donor will be compared among

each other, as well as with the original human sample used as the inocula. This result will confirm successful establishment of the human microbiota in the rat hosts.

Once the establishment of the human microbiota in the rat hosts is confirmed, two humanized rats derived from each human donor will be randomly selected and group-housed with the humanized rats derived from the other human donors. This step is to allow the animals to transfer their gut microbiota among each other, resulting in a unified de-individualized population of the human gut microbiota. To test the reproducibility of this approach, two such groups of communities of rats will be generated and housed separately.

The establishment of a unified de-individualized human microbiota and the stability of the established gut microbiota in our DHR model will be studied by metagenomics, using fecal microbiota. Fresh fecal samples will be collected at four time points (i.e. 2, 4, 8, and 16 weeks) after the start of group housing, and microbial DNAs isolated from the fecal materials.

The V1-V3 region of the microbial 16S rRNA gene will be amplified for multiplex sequencing. Redundant/duplicate reads will be removed with their total counts recorded for determination of the abundance of the respective sequences. The sequencing data will be converted to “fasta” (or “qual”) files for database searches. Family assignments will be based on the NCBI taxonomic tree. Clustering of samples by taxonomy will be performed using the MEGAN (Metagenome Analyzer) software.

The functional stability of the human microbiota in the DHR Model will be investigated using metatranscriptomics. Microbial RNAs extracted from fresh fecal samples will be divided into two aliquots, one with and the other without microbial rRNA depletion, prior to cDNA synthesis. cDNA will be synthesized in the presence of rRNA-specific primers or random primers (for the rRNA-depleted samples), and the resulting cDNAs amplified using the GenomiPHI DNA Amplification Kit. Shotgun sequencing of the amplified cDNAs will be performed, and taxonomical classification and species/subspecies abundance based on the rRNA sequencing data, as above. The results will be compared with the metagenomics results to determine if the numerically dominant members in the microbiota community also show metabolic dominance.

The mRNA sequencing datasets will be annotated by searching against the NCBI non-redundant protein database using BLASTX. Clustering analyses will be performed on both the nucleotide sequences and the translated peptide sequences using the CD-HIT software with a two-step approach. The first step will cluster sequences at 95% identity over 80% of sequence length to identify non-redundant sequences. The total counts of redundant reads will be recorded for the determination of the abundance of the respective sequences. At the second step, the non-redundant sequences will be clustered at 60% identity, over 80% of sequence coverage, to produce clusters of protein homologs/families. To functionally categorize the expressed genes, the translated peptide sequences will be assigned to functional groups by querying the Clusters of

Orthologous Groups (COG) and the Kyoto Encyclopedia for Genes and Genomes (KEGG) databases. A hypergeometric enrichment test will be performed to identify over-representation of expressed genes in the orthologous groups.

The DHR animals will be compared with the SPF rats for intestinal microbial growth profile at the time points outlined above. Serum hormones related to the HPA axis functions (e.g. adrenocorticotropic hormone (ACTH) and corticosterone), will be assessed using ELISA. In addition, serum profile of selected cytokines will be quantified to assess up-regulation of proinflammatory cytokines and/or down-regulation of anti-inflammatory cytokines. Plasma endotoxin load (i.e. the level of LPS-binding protein) will also be determined and correlated with the cytokine profiles. These results will reveal if there is any gross abnormality of our DHR Model.

5.0 Conclusion

We have initiated an investigation to study the effects of sleep deprivation, a frequent stressor during military missions, on the gut microbiome population. We hypothesize that sleep deprivation causes an imbalance in the gut microbiome composition, consequently altering intestinal permeability and leakage of microbial cells and inflammatory factors that ultimately dysregulate the HPA axis. Knowledge gained in this study will provide the basis for the development of novel strategies to alleviate the physiological and secondary downstream stresses caused by sleep deprivation.

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